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Mouse mammary tumor viruses (MMTVs) encode superantigens that associate with major histocompatibility complex class II products on antigen-presenting cells and stimulate T cells in a Vβ-specific manner. This T cell activation is critical for completion of the viral life cycle and vertical transmission to the next generation. To investigate the functional significance of extensive viral superantigen (Sag) glycosylation, we disrupted the six potential sites for N-linked carbohydrate addition in the Sag encoded by proviral integrant *Mtv-1.* Shifts in the apparent molecular mass of these mutant glycoproteins suggested that wild-type *Mtv-1* Sag is glycosylated on four of its six sites. Intracellular and cell surface staining of the panel of mutants indicated that any decrease in glycosylation resulted in reduced levels of intracellular protein and undetectable surface expression, suggesting that decreased glycosylation leads to rapid Sag degradation and abates trafficking to the plasma membrane. Nevertheless, several mutants with intermediate levels of glycosylation expressed enough Sag on the B cell surface to potently stimulate reactive T cell hybrids. We show there is no specific site bearing N-linked glycosylation that is essential for activity, but at least one carbohydrate addition is necessary for effective B cell presentation of MMTV superantigens to T cells.

INTRODUCTION

The mouse mammary tumor virus 3′ long terminal repeat is unusual in that it contains an open reading frame. The encoded glycoprotein has been dubbed a viral "superantigen" (Sag) because of its surprising ability to stimulate a large fraction of the murine T cell repertoire (Frankel *et al.,* 1991; Dyson *et al.,* 1991; Choi *et al.,* 1991). Superantigens are distinguished from conventional antigens by binding to major histocompatibility complex (MHC) class II products in a region distinct from the polymorphic peptide binding groove, as well as by being presented by class II$^+$ cells without extensive proteolytic processing (Mottershead *et al.,* 1995; Dellabona *et al.,* 1990; Yagi *et al.,* 1990). The Sag/class II complex stimulates T cells bearing particular T cell receptor (TCR) Vβ elements (Herman *et al.,* 1991).

Both MMTV infectious viruses and endogenous proviruses produce Sags (Acha-Orbea *et al.,* 1993). All MMTV Sags share a high degree of sequence homology, except at the carboxy terminus, which is the region that determines TCR Vβ specificity (Yazdanbaksh *et al.,* 1993). Thus, different Sags are able to stimulate distinct subsets of the T cell repertoire.

The Sag glycoprotein plays a crucial role in the life cycle of MMTV. Milk-borne virus infects B cells in the Peyer's patches of suckling pups, followed by retroviral integration and expression of Sag with MHC class II products on the B cell surface (Karapetian *et al.,* 1994). Presentation of Sag results in the stimulation of reactive helper T cells, which induce differentiation and extensive clonal expansion of the infected B cells (Chervonsky *et al.,* 1995; Held *et al.,* 1994, 1993a). In mice lacking Sag-reactive T cell subsets, MMTV infection is prevented (Held *et al.,* 1993b; Golovkina *et al.,* 1992). Migration of infected lymphocytes is presumably responsible for the eventual infection of the mammary epithelium.

MMTV integration into germ cells has resulted in *Mtv* proviral integrants that are numerous in all inbred strains and most wild mice (Pullen *et al.,* 1990a; Kozak *et al.,* 1987). While most of these integrants have lost the ability to make infectious virus, their Sag expression during ontogeny leads to the deletion of reactive thymocytes (Herman *et al.,* 1991). This Vβ-specific deletion profoundly effects the T cell repertoire and immune surveillance (Lukacher *et al.,* 1995; Pullen *et al.,* 1988).

Although TCR residues have been identified that contribute to Sag recognition (Pullen *et al.,* 1991, 1990b; Cazenave *et al.,* 1990), the biochemistry and cell surface presentation of Sag is less well understood. Sags are

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type II glycoproteins, with a short intracellular amino-terminal domain, a membrane-spanning region, and a large extracellular carboxy-terminal domain (Knight *et al.,* 1992; Korman *et al.,* 1992; Choi *et al.,* 1992). On the cell surface, Sags are found predominantly as a proteolytic cleavage product (Park *et al.,* 1995; Winslow *et al.,* 1992), perhaps due to processing at one or more subtilisin-like cleavage sites. Such processing may be similar to what occurs in a variety of cellular and viral surface glycoproteins, including the insulin proreceptor and all retroviral envelope glycoproteins (Dickson *et al.,* 1982; Ullrich *et al.,* 1985). It has been previously shown that Sags are heavily glycosylated in B cells, and much, if not all, of the carbohydrate addition is in the form of asparagine-linked oligosaccharide (Winslow *et al.,* 1994). Studies employing PNGase F (*N*-glycanase) and EndoH digestion of Sags have suggested that the predicted 37-kDa core protein is initially increased to a molecular mass of 45 kDa by cotranslational N-linked oligosaccharide addition in the endoplasmic reticulum (ER) (Brandt-Carlson and Butel, 1991; Korman *et al.,* 1992). Further variable carbohydrate modifications in the Golgi complex, possibly including O-linked glycosylation, result in a mature glycoprotein of variable molecular mass ranging up to approximately 82 kDa (Winslow *et al.,* 1994). Thus, carbohydrate can comprise up to 55% of the Sag glycoprotein mass.

Carbohydrate addition can fulfill several possible functional roles for a glycoprotein, including facilitating correct intracellular trafficking, solubility, resistance to proteolysis, and ligand binding. In addition, for many cell surface receptors N-linked glycosylation has been shown to be required for attaining proper conformation during folding in the ER (Hoe and Hunt, 1992; Olson and Lane, 1987; Slieker *et al.,* 1986). Conversely, glycosylation appears unnecessary for the trafficking and function of many secreted glycoproteins (West, 1986). Our earlier studies indicated that preventing TCR glycosylation actually improved Sag binding (Pullen *et al.,* 1991), and a similar effect has been reported for IgM binding of a cognate ligand (Bazin *et al.,* 1992).

The *Mtv-1* provirus, found in several inbred mouse strains, encodes the Vβ3-reactive viral superantigen *Mtv-1* Sag (Pullen *et al.,* 1992). The amino acid sequence of *Mtv-1* Sag includes six potential sites for N-linked glycosylation (the amino acid motif N-X-S/T), all of which lie in the extracellular domain. Five of these sites (N79, N89, N93, N131, and N146) are conserved among infectious MMTVs and proviral integrants (Brandt-Carlson *et al.,* 1993). To address the functional significance of *Mtv-1* Sag carbohydrate addition, we performed site-directed mutagenesis on the six motifs. A panel of mutants containing disruptions in combinations of these motifs was generated, followed by characterization of their biochemical and functional properties. B lymphocytes are the central Sag-presenting cells *in vivo* (Held *et al.,* 1993a,b); therefore all constructs were transfected into the B cell line CH27. We found that any decrease in the number of oligosaccharide additions drastically diminished the amount of *Mtv-1* Sag expressed on the cell surface. A construct with all six sites disrupted encoded a Sag that was no longer presented to most Vβ3$^+$ T cell hybrids, demonstrating that some level of N-linked glycosylation is required for effective MMTV viral superantigen function. The data obtained suggest that one N-linked oligosaccharide addition on *Mtv-1* Sag molecules can be sufficient to stimulate all Vβ3$^+$ T cell hybrids tested.

MATERIALS AND METHODS

Mutagenesis and plasmids

The mutagenesis strategy employed is shown in Fig. 1A. The sequences of oligonucleotide primers are shown in Table 1. To disrupt glycosylation sites at amino acids 79, 80, 89, and 93, PCR was first used to generate a gene fragment between primers 5′ sag1 and 3′ 79–93, using the cloned *Mtv-1 sag* gene (Pullen *et al.,* 1992) as a template. Primer 3′ 79–93 introduces one N→S and three N→Q amino acid changes and a unique *Xho*I site. Another PCR used primers 5′ 79–93, which introduces the same *Xho*I site, and 3′ sag1. These PCR fragments were separated in a low-melt agarose gel, and 2-μl quantities from each of these bands were then used together as the templates for sequence overlap extension PCR (Ho *et al.,* 1989), using primers 5′ sag1 and 3′ sag1. The resulting full-length PCR product was digested with *Eco*RI and *Bam*HI, cloned into pBluescript(SK$^{+/-}$) phagemid (Stratagene, La Jolla, CA), and sequenced in its entirety to confirm the desired mutations. Since primer 5′ 79–93 does not encode disruptions in the glycosylation sites, clones were also obtained that lacked disrupted sites, but included the introduced *Xho*I restriction site. These clones made it possible to subclone regions encoding one or two wild-type glycosylation sites into mutant constructs without additional PCR. A similar approach was used to disrupt glycosylation sites at amino acids 131 and 146, using the 131–146 primer set. Using the introduced *Xho*I and *Xma*III restriction sites and a unique *Bsm*I site found in the *Mtv-1 sag* nucleotide sequence, a panel of constructs was generated that encodes mutant Sags with some or all of the glycosylation sites disrupted, as represented in Fig. 1B. These constructs were subsequently subcloned into the pHβAPr-1 expression vector, which employs the β-actin promoter and confers G418 resistance (Gunning *et al.,* 1987).

Cell lines and transfection

Transfectants of the B cell lymphoma CH27 (H-2^a) (Haughton *et al.,* 1986) expressing wild-type and mutant *Mtv-1* Sags were generated by linearizing the expression

constructs described above with *Sca*I, followed by electroporation with a Promega X-Cell 450 electroporator (375 V, 1400 μF, 50 msec) (Madison, WI). Transfected cells were cloned at limiting dilution and selected with 1.3 mg/ml G418. 10–40 clones from each transfection were screened in stimulation assays using Vβ3$^+$ T cell hybrids K25-59.6, K25-49.16 (Pullen *et al.,* 1988), and 5KC-73.8.11 (White *et al.,* 1993). The Vβ7$^+$ hybrid KOX7-6.6 (Choi *et al.,* 1991) served as a negative control.

Affinity purification and Western blotting

Affinity purification and Western blot analysis of *Mtv-1* Sag was carried out, with minor modifications, as previously described for *Mtv-7* Sag (Winslow *et al.,* 1994). Briefly, VS1, a mAb recognizing the amino terminus of all MMTV-encoded superantigens, was conjugated to cyanogen bromide-activated Sepharose. CH27 cells expressing mutant *Mtv-1* Sags were lysed at 10^8/ml in PBS containing 1% NP-40, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μ*M* pepstatin, 30 m*M* iodoacetamide, 0.1% NaN_3, 5 m*M* EDTA, 100 μ*M* TLCK, and 0.5 m*M* PMSF (all from Sigma Chemical Co., St. Louis, MO). After rocking for 1 hr at 4°, lysates were clarified by centrifugation for 1 hr at 64,000 *g.* Clarified lysates were run over the VS1 affinity column at 4°, and the column was washed with PBS/ 0.5% NP-40/ 0.1% SDS, followed by 2 m*M* NH_4HCO_3. Bound material was eluted with 50 m*M* diethylamine and was lyophilized. After resuspension in a small volume of 0.5% SDS/ 1% 2ME, samples were treated with either 5 or 1.5 μl PNGase F (New England Biolabs, Beverly, MA) overnight.

For Western analysis, VS1-column eluate corresponding to 2×10^8 cell equivalents (per lane) was electrophoresed in 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane. Blots were blocked in PBS containing 5% nonfat dry milk and 0.1% Tween 20. Sag protein was probed with VS1 (10 μg/ml), with or without 12 μ*M* competing peptide (Winslow *et al.,* 1994). A mouse anti-Armenian hamster IgH monoclonal antibody, HIG632.13 (Winslow *et al.,* 1992), was coupled to activated digoxigenin ester (Boehringer Mannheim, Indianapolis, IN) at 100 μg DIG/mg mAb and was used at 0.2 μg/ml, followed by anti-DIG-peroxidase Fab (1:8000, Boehringer Mannheim). All washes and antibody incubations were carried out at room temperature in PBS with 3% milk and 0.1% Tween 20. The ECL chemiluminescence system (Amersham Life Science Inc., Arlington Heights, IL) was used for visualization. The VS1-2.8 cell line was a generous gift of Dr. Gary Winslow (Wadsworth Center for Laboratories and Research, Albany, NY).

Antisera production

Peptide P2 (NH_2-CYNSREEAKRHIIEHI) was synthesized and purified by the Molecular Pharmacology Facility at the University of Washington. This peptide corresponds to amino acids 294–308 of *Mtv-1* Sag, with the addition of a cysteine residue to facilitate covalent coupling to KLH using an Imject conjugation kit (Pierce Chemical Co., Rockford, IL). New Zealand White rabbits were injected sc with 400 μg of P2/KLH conjugate emulsified with CFA. Four subsequent injections were made using IFA. Antisera were tested by ELISA for P2 reactivity (data not shown) and by flow cytometric analysis for binding to CH27 cells transfected with *Mtv-1* Sag.

Flow cytometric analysis

To block antibody binding to Fc receptors, cells were preincubated for 15 min at 37° in an anti-FcγRII/III hybridoma supernatant (2.4G2) (Unkeless, 1979) containing 0.1% NaN_3. Rabbit anti-*Mtv-1* Sag serum or prebleed serum was then added at a final dilution of 1:300, and incubation was continued for 1 hr. Where indicated, competitor P2 peptide was added at 25 μ*M.* Secondary and tertiary antibodies were mouse anti-rabbit Ig (Pierce) coupled to digoxigenin as above and sheep anti-digoxigenin-FITC Fab (Boehringer Mannheim), respectively. Incubations were for 30 min on ice.

For intracellular staining, cells were preincubated 10 min in 50% normal mouse serum and 50% 2.4G2 supernatant containing 0.03% saponin (Sigma). Biotinylated VS1 mAb was added at 3 μg/ml (30 min, 25°) with or without 25 μ*M* competitor peptide, followed by tricolor-coupled streptavidin (Caltag Laboratories, So. San Francisco, CA). The reproducibility of permeabilization and intracellular staining was assessed with In1, a mAb recognizing invariant chain (Koch *et al.,* 1982). Incubation with In1 supernatant was followed with FITC-mouse anti-rat F(ab′)$_2$ (Accurate Chemical and Scientific, Westbury, NY). Washes and incubations were in PBS containing 1% BSA, 0.1% NaN_3 and 0.03% saponin. All samples were analyzed using a FACScan (Becton–Dickinson & Co., Sunnyvale, CA) and ReproMan software (TrueFacts Software Inc., Seattle, WA).

T cell stimulation assay

Stimulation assays were carried out as previously described (Morishima *et al.,* 1994). Briefly, 10^5 CH27 cells or *Mtv-1* Sag transfectants were cocultured with 10^5 T cell hybrids for 24 hr at 37°. The supernatants of these cultures were assayed for lymphokine production with the IL-2-dependent cell line, HT-2 (Kappler *et al.,* 1981). Survival of HT-2 cells was measured using alamarBlue (Alamar Biosciences Inc., Sacramento, CA). Values presented in Table 2 are averages of triplicate wells. Each transfectant was assayed in at least three independent experiments. The data from one representative experiment are shown.

TABLE 1

Oligonucleotide Primers Employed in These Studies

Primer	Sequence (5′–3′)
5′sag1	GAAGAAGCTTGAATTCATGCCGCGCCTGCAG
3′sag1	ATGCGGATCCAAAGCTAAGGGCAAA
3′sag1/6	GTCTTTTGGCCTCCTCTCTACT
5′79–93	CTTGATTCTTTTAATAACTCGAGCGTGCAAGATTAC
3′79–93	GAGGAAGGTCGACTGCTCCGAATCCTGTAGATTGTAATCTTGCACGCTCGAGCTCTGAAAAGAATCAAG
5′131–146	TATATTTTTACCAATGAGACCAATCCAATCGGCCGATTATTAATC
3′131–146	AGACAAAGATTCCTGTCTTAACATCATGATTAATAATCGGCCGATCGGATTGGTCTCCTGGGTAAAAATATA
5′HPRT	GTTGGATACAGGCCAGACTTTGTTG
3′HPRT	GAGGGTAGGCTGGCCTATGGCT

Quantitation of Mtv-1 Sag-specific mRNA

RNA was isolated from stable transfectants using RNAzol B (Tel-Test, Inc., Friendswood, TX), and cDNA was synthesized using oligo(dT)$_{12-18}$ primer and Superscript II reverse transcriptase (GibcoBRL, Gaithersburg, MD). Primers specific for *Mtv-1 sag* (5′ sag1 and 3′ sag1/6, Table 1) were used for PCR amplification. Threefold serial dilutions of cDNA served as PCR templates, and the most dilute sample yielding a detectable PCR product was considered the *Mtv-1* Sag mRNA titer. These titers were then normalized to HPRT-specific PCR reactions carried out upon cDNA from each mutant, using the same titrations and conditions, to control for overall cDNA levels among samples (Reiner *et al.,* 1993). All dilutions and PCRs were repeated at least once. Transfectants with similar *Mtv-1* Sag mRNA levels were chosen for further study.

RESULTS

Disruption of potential N-linked glycosylation sites

We employed site-directed mutagenesis to determine the role of N-linked glycosylation in the generation of functional MMTV-encoded superantigens. Sequence overlap extension PCR was used to introduce mutations in various combinations of the six potential sites for N-linked oligosaccharide addition on *Mtv-1* Sag (Fig. 1A). At five of the six positions, Asn residues of the N-X-S/T motif were replaced with Gln. This conservative Asn→Gln substitution should have minimal effect on protein tertiary structure while preventing oligosaccharide transfer. At position 80, an Asn→Ser change was made because this substitution mimics the serine residue found at the same position in *Mtv-7* Sag (Beutner *et al.,* 1992). Mutants with various disrupted glycosylation motifs are represented in Fig. 1B.

mRNA and Western blot analysis of *Mtv-1* Sag mutants

Wild-type *Mtv-1* Sag and the mutant constructs described above were cloned into an expression vector under the control of the human β-actin promoter and were transfected into the B cell line CH27. Stable transfectants with high levels of *Mtv-1* Sag-specific mRNA were selected for further analysis. Semiquantitative RT–PCR confirmed that the wild-type and all mutant

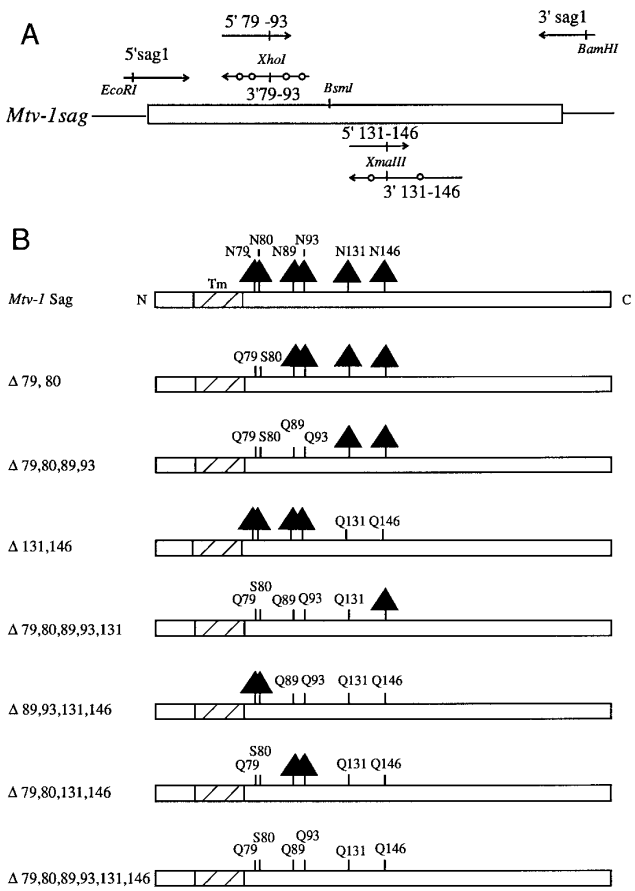

FIG. 1. *Mtv-1* Sag mutants lacking sites for N-linked glycosylation. (*A*) Primers used to introduce mutations via sequence overlap extension PCR. Introduced restriction sites are labeled on the primers, and nucleotide changes that result in disruption of glycosylation sites are denoted with open circles. (*B*) Panel of mutant *Mtv-1* Sag proteins. Remaining sites for potential N-linked addition are represented as triangles.

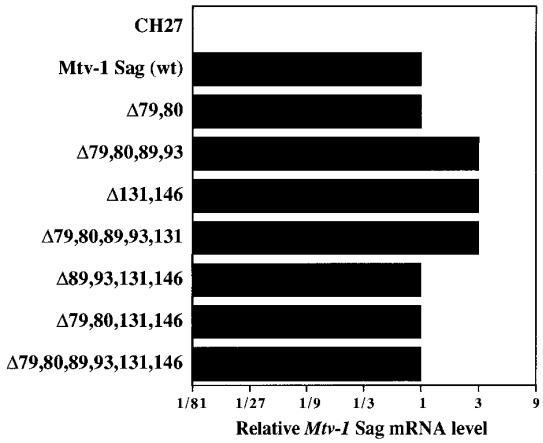

FIG. 2. *Mtv-1* Sag mRNA levels of transfectants. RNA was isolated from clones of stable transfectants and was reverse transcribed. Threefold serial dilutions of these cDNAs served as templates for PCRs using *Mtv-1 sag*-specific primers. The last template dilution to yield a PCR product was considered the cDNA titer. To control for variation between cDNA preparations, these Sag mRNA titers were normalized using HPRT-specific PCRs of the same cDNA serial dilutions. Sag mRNA levels of the mutant transfectants are shown relative to the wild-type *Mtv-1* Sag transfectant.

Mtv-1 Sag transfectants used in subsequent experiments expressed similar levels of *Mtv-1* Sag mRNA (Fig. 2).

Cell lysates were run over a VS1 affinity column that binds the Sag N-terminus. Material eluted from this column was in some cases treated with PNGase F (*N*-glycanase), followed by SDS–PAGE, transfer to nitrocellulose, and immunoblotting with the same mAb. The enrichment step preceding Western analysis has been shown to be necessary; despite the fact that the constructs are expressed by a strong promoter, approximately 2×10^8 cell equivalents are needed to detect Sag protein by immunoblotting (Winslow *et al.,* 1992, and our unpublished data). Although VS1 also recognizes endogenous Sags expressed in the CH27 cell line, these Sags are expressed at almost undetectable levels, allowing analysis of transfected mutants (Fig. 3). Moreover, these endogenous Sags are not Vβ3-reactive and therefore did not interfere with stimulation assays using V$\beta3^+$ T hybrids.

Following treatment with PNGase F, wild-type *Mtv-1* Sag and all mutants with detectable levels of protein migrated as a doublet of 37 and 39 kDa (Fig. 3). p37 corresponds to the predicted core molecular mass of *Mtv-1* Sag (36.5 kDa); although VS1 cross-reacts with a faint 37-kDa species in all lanes, *Mtv-1* Sag is conspicuous in its intensity. The 39-kDa band most likely represents incomplete digestion by PNGase F, because this band, and bands of greater molecular mass, increased in intensity with less exhaustive digestion (data not shown). The 32-kDa protein is PNGase F, which was detected by the secondary antibody in cases when more enzyme was used to maximize oligosaccharide removal.

Without PNGase F treatment, wild-type *Mtv-1* Sag glycoprotein migrates predominantly as a band of molecular mass of 45 kDa (gp45), as previously reported for other Sags (Korman *et al.,* 1992; Brandt-Carlson and Butel, 1991; Krummenacher and Diggelmann, 1993). EndoH treatment has identified gp45 as the ER-associated form of the glycoprotein that is cotranslationally modified with core oligosaccharides (Winslow *et al.,* 1994). Each of the high-mannose moieties adds approximately 2 kDa to the molecular mass of an immature glycoprotein; therefore, the decreased mobility of deglycosylated *Mtv-1* Sag (8 kDa) suggests that the wild-type glycoprotein is modified with four N-linked oligosaccharide additions. Mutant Δ79,80 also migrates at a molecular mass of 45 kDa, suggesting that N-linked glycosylation is occurring at all four remaining sites in this mutant.

Mutant Δ79,80,89,93 yields a prominent band of 41 kDa (gp41), corresponding to a deficit of two high-mannose oligosaccharides with respect to wild type and a more faint band at 39 kDa. This result suggests that both amino acids N131 and N146 are generally glycosylated, but that perhaps some of the glycoprotein is glycosylated at only one of these sites. Mutant Δ131,146 also yields a prominent gp41 moiety, indicating that only two of the remaining four motifs (N79, N80, N89, N93) are glycosylated in this mutant. The increased mobility observed with these mutants is not the result of proteolytic degradation, because treatment of these glycoproteins with PNGase F yields predominantly p37 in all cases.

Sag protein cannot be detected by Western analysis in the remaining mutants (Δ79,80,89,93,131, Δ89,93, 131,146, Δ79,80,131,146, and Δ79,80,89,93,131,146), and therefore the glycosylation status of these mutants can-

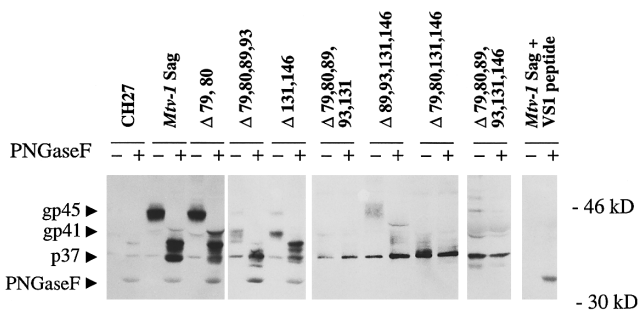

FIG. 3. Western blot analysis of mutants. *Mtv-1* Sag was purified from clarified cell lysates over a VS1 affinity column, run on SDS–PAGE, transferred to nitrocellulose membrane, and blotted with VS1. *PNGase F+* indicates sample was treated with 5 or 1.5 μl *N*-glycanase prior to electrophoresis. Note that in samples where 5 μl PNGase F was used, the protein is detected on the blot. This is the only band seen on membranes blotted with VS1 in the presence of 12 μM competitor peptide. The maturing, post-ER glycoproteins, which migrate as faint species of heterogeneous molecular mass (50–82 kDa), and the 24-kDa putative proteolytic cleavage products ((Winslow *et al.,* 1994), data not shown) that were detected inconsistently, are not included in the figure.

not be determined directly. This result is most likely due to the low levels of protein present in these cells, as suggested by intracellular staining (Fig. 4) and as discussed in more detail below. In summary, the mutants with detectable shifts in molecular mass suggest that wild-type *Mtv-1* Sag is generally glycosylated at amino acids N131 and N146 and at two of the four remaining motifs, resulting in a total of four N-linked oligosaccharide additions.

Cell surface and intracellular expression of *Mtv-1* Sag mutants

Rabbits were injected with peptide/ KLH conjugates to generate antisera specific for the extracellular carboxy terminus of *Mtv-1* Sag. One of the antisera obtained readily allowed detection of wild-type *Mtv-1* Sag on the surface of transfected CH27 cells by flow cytometry (Fig. 4). However, surface expression of all glycosylation mutants was undetectable, with the exception of trace staining reproducibly seen on mutant Δ79,80. To rule out the possibility that reduced N-linked glycosylation of mutants might affect epitopes recognized by the anti-*Mtv-1* Sag antiserum, the antiserum was shown to effectively block *in vitro* stimulation of Vβ3$^+$ T cell hybrids by CH27 cells expressing stimulatory mutant constructs (data not shown). Therefore, we conclude that even a modest reduction in glycosylation results in a marked decrease in *Mtv-1* Sag surface expression.

In contrast, Sag protein was detectable by intracellular VS1 staining of saponin-permeabilized cells expressing mutant Sags, with the exception of the mutant lacking all potential N-linked glycosylation sites (Fig. 4). In most mutants, the intracellular levels of Sag were greatly reduced compared to wild type. This reduction was not due to a decrease in *Mtv-1* Sag-specific mRNA, since mRNA levels between the mutants and wild-type do not differ significantly (Fig. 2). One mutant, Δ131,146, had almost wild-type levels of intracellular Sag and yet lacked detectable cell surface expression.

Functional effects of abrogating N-linked glycosylation

CH27 cells transfected with mutant constructs were assayed for the ability to stimulate a panel of T cell hybrids. As seen in Table 2, Vβ3$^+$ T cell hybrids secrete high levels of IL-2 when incubated with CH27 cells expressing wild type *Mtv-1* Sag. However, cells expressing the *Mtv-1* Sag mutant lacking all N-linked glycosylation motifs (Δ79,80,89,93,131,146) did not stimulate most Vβ3$^+$ T cell hybrids (Table 2 and data not shown). Several other mutants with intermediate numbers of ablated N-linked glycosylation sites stimulate all Vβ3$^+$ T cell hybrids tested, indicating that full glycosylation of *Mtv-1* Sag is not necessary for function. Most notably, Δ78,80,89,93,131, which can only be glycosylated on amino acid 146, has significant stimulatory ability, demonstrating that in this instance one N-linked addition is adequate for presentation to all of the Vβ3$^+$ T cell hybrids tested. Not all mutant Sag proteins that can be glycosylated at one or more sites exhibit this level of activity, however. Two mutants that are potentially glycosylated at two sites (Δ89,93,131,146 and Δ79,80,131,146) elicit little or no IL-2 production by the *Mtv-1* Sag-reactive hybrids. One extremely sensitive T cell hybrid, 5KC-73.8.11, secretes low levels of IL-2 in response to mutants that cannot stimulate any of the other Vβ3$^+$ hybrids. This is probably a reflection of these mutants yielding a trace amount of functional *Mtv-1* Sag protein on the cell surface.

It should be noted that mutants Δ79,80,89,93 and Δ131,146 both stimulate IL-2 production by Vβ3$^+$ hybrids. Since these two mutants encompass the disruption of all possible glycosylation motifs, this suggests that there is no particular site for carbohydrate addition that is critical for *Mtv-1* Sag function. In addition, these results imply that none of the amino acid changes grossly perturb *Mtv-1* Sag conformation, since both of these mutants are effectively presented to T cells. Therefore, we infer that the defect of the nonstimulatory mutants is due to their glycosylation status.

DISCUSSION

The functional role of MMTV-encoded superantigens in promoting proviral amplification during infection and the requirement for Vβ-specific stimulation for viral transmission to the next generation is well established (Acha-Orbea and MacDonald, 1995). However, several aspects of Sag trafficking, processing, and cell surface presentation remain poorly understood. In this report, we address the importance of the extensive and variable N-linked oligosaccharide modification of Sags. We show that preventing glycosylation at increasing numbers of potential N-linked sites results in increasingly poor intracellular *Mtv-1* Sag stability, surface expression, and stimulation of reactive T cell hybrids. In the most extreme case, *Mtv-1* Sag lacking any N-linked glycosylation cannot be detected within the cell or on the cell surface by staining or biochemical means, and the extremely low levels of this mutant protein can only be detected by one particularly sensitive Vβ3$^+$ T cell hybrid.

There are several possible explanations for why Sags require N-linked glycosylation to be presented correctly to T cells. Carbohydrate may directly contribute to the Sag interaction with MHC class II or the TCR. However, since prevention of any individual N-linked oligosaccharide addition does not eliminate T cell recognition, our data indicate that such carbohydrates are unlikely to play an essential role in Sag binding to these molecules. Alternatively, perhaps glycosylation protects the glycoprotein

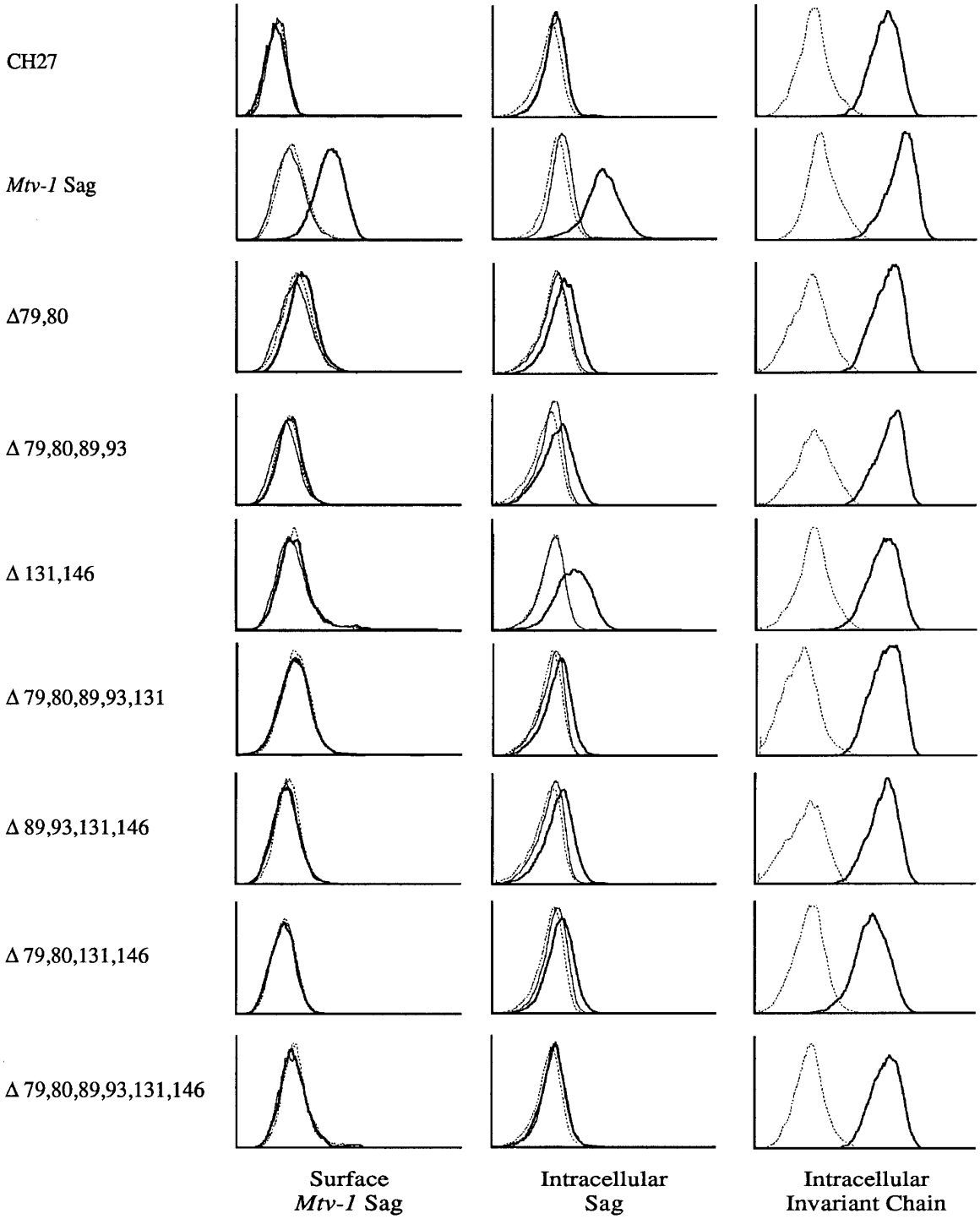

FIG. 4. Cell surface and intracellular expression of *Mtv-1* Sag mutants. To measure *Mtv-1* Sag surface expression, transfectants were stained with anti-*Mtv-1* Sag antiserum, in the presence (plain lines) or the absence (bold lines) of competitor peptide P2 or with prebleed serum (dashed lines). For intracellular staining, transfectants were permeabilized with saponin and stained with biotinylated VS1 mAb, in the presence (plain lines) or the absence (bold lines) of competitor peptide or with no primary Ab (dashed lines). To detect intracellular invariant chain, permeabilized transfectants were stained with In1 mAb (bold lines) or secondary antibody only (dashed lines). Fluorescence intensity is represented on a logarithmic scale on the *x*-axis.

TABLE 2

Stimulation of T Cell Hybridomas by *Mtv-1* Sag Glycosylation Site Mutants

	IL-2 production by T cell hybrids (U/ml)			
	K25-59.6 (Vβ3$^+$)	5KC-73.8.11 (Vβ3$^+$)	K25-49.16 (Vβ3$^+$)	K0X7-6.6 (Vβ7$^+$)
CH27	—[a]	—	—	—
Mtv-1 Sag (wt)	1600	796	768	—
Δ79, 80	341	599	473	—
Δ79, 80, 89, 93	129	296	217	—
Δ131, 146	355	849	491	—
Δ79, 80, 89, 93, 131	68	231	83	—
Δ89, 93, 131, 146	5	39	9	—
Δ79, 80, 131, 146	—	7	—	—
Δ79, 80, 89, 93, 131, 146	—	15	—	—

[a] — Indicates $\leq$ 5U IL-2/ml.

from proteolytic degradation during trafficking and presentation. This possibility seems especially plausible if Sag traffics together with MHC class II via protease-rich endocytic compartments. We feel, however, that in most cases the most straightforward explanation for the attenuated Sag presentation is that the glycosylation mutants are improperly folded in the ER and are degraded more rapidly than wild-type *Mtv-1* Sag protein.

N-linked glycosylation has been shown to be important for the proper folding and trafficking of many cell surface glycoproteins, including the GLYT1 glycine transporter and the transferrin receptor (Williams and Enns, 1993; Olivares *et al.,* 1995). High-mannose oligosaccharides appear to play a role in early glycoprotein association with molecular chaperones such as calnexin and BiP, which guide stable folding in the ER (Ware *et al.,* 1995). Improperly folded proteins are retained in the ER, and are quickly destroyed. Moreover, MMTV Sags are rich with Pro, Glu, Ser, and Thr residues ("PEST" sequences) that earmark proteins for rapid degradation (Rechsteiner, 1990; Stellwagen, 1992; Rogers *et al.,* 1986), and MMTV(GR) Sag has been demonstrated to have a short half-life of less than 2 hr *in vitro* (Krummenacher and Diggelmann, 1993). It therefore seems likely that improperly folded glycosylation mutants would be extremely short-lived and would only be present at low levels.

It is noteworthy that the majority of Sag glycoprotein detected by immunoblotting is EndoH-sensitive and is inferred to reside in the ER (Winslow *et al.,* 1994). Therefore, the decreased levels of intracellular Sag mutants observed with flow cytometry suggest that the mutant glycoproteins are subject to degradation before leaving the ER. An intriguing exception to this trend is found in mutant Δ131,146, which exhibits near wild-type levels of intracellular Sag, yet *Mtv-1* Sag cannot be detected on the cell surface. In this case the lack of mutant *Mtv-1* Sag surface expression may not be a direct consequence of protein instability, but may instead be a reflection of improper targeting and transport from the ER.

It is interesting that any decrease in glycosylation results in cell surface *Mtv-1* Sag levels that are undetectable by staining, and yet many of these mutants are able to effectively stimulate T cells. This apparent discrepancy may be reconciled by the observation that T cell hybrids secrete IL-2 in response to extremely low levels of presented antigen (Harding and Unanue, 1990). We have also observed that T cell stimulation is more sensitive than surface staining as a method for detecting Sags expressed by LPS-stimulated B cell blasts (data not shown). In addition, after extended periods in culture, variants of CH27 transfectants expressing wild-type *Mtv-1* Sag can be isolated; these variants also stimulate Vβ3$^+$ hybrids to produce IL-2 in the same range as the glycosylation site mutants, despite having undetectable *Mtv-1* Sag surface expression by flow cytometry (data not shown). These results have important implications for MMTV infection, as they demonstrate that Vβ-specific T cell activation is exquisitely sensitive to low level Sag expression.

Several observations suggest that use of individual glycosylation sites on *Mtv-1* Sag mutants may differ depending upon which combination of sites are available. For instance, although mutant Δ79,80 and wild-type *Mtv-1* Sag have the same apparent molecular mass, they differ in *Mtv-1* Sag surface expression (Fig. 4) and their ability to stimulate reactive T cell hybrids (Table 2). One possible interpretation is that mutant Δ79,80 and wild-type *Mtv-1* Sag are glycosylated at different sites, despite having the same number of carbohydrate additions. Alternatively, it is formally possible that in wild-type *Mtv-1* Sag residues N79 and N80 are not glycosylated, but may contribute to folding, interaction with MHC class II, or intracellular trafficking.

The functional data also have implications for the mu-

tants that are not detected by immunoblotting (Fig. 3). For example, we infer that mutant Δ79,80,89,93,131 is most likely glycosylated at amino acid N146, since this mutant is able to effectively stimulate all the Vβ3$^+$ hybrids, while the mutant lacking all N-linked glycosylation sites, Δ79,80,89,93,131,146, stimulates only poorly the most sensitive hybrid.

We have studied the impact of N-linked glycosylation on Sag presentation to understand better how MMTV utilizes viral superantigens to exploit the murine immune system. We show that extensive glycosylation is necessary for high levels of *Mtv-1* Sag surface expression on antigen-presenting cells. However, several mutants with intermediate levels of glycosylation express levels of *Mtv-1* Sag on the B cell surface sufficient to stimulate reactive T cell hybrids. While addition of a single N-linked carbohydrate can confer significant activity, ablation of all glycosylation sites results in a poorly functional Sag that is unable to stimulate most members of a panel of reactive T cell hybrids. Taken together, the data presented indicate that effective presentation of MMTV superantigens requires N-linked carbohydrate addition.

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